Nonaqueous Beef Flavor Components. Composition of Petroleum Ether–Extractable Intramuscular Polar Lipids

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Petroleum ether–extractable intramuscular lipids were separated by silica gel chromatography into polar and neutral lipids. Polar lipids were fractionated on an anion exchange DEAE-cellulose column into at least 11 components. Thin-layer chromatography R_f values, staining behavior with specific reagents, and infrared spectra of intact lipids enabled the identification of seven polar lipids. Six of these were positively identified as phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, and phosphatidylinositol; the seventh compound was tentatively identified as sulfatide.

Voluminous work has been reported on polar lipids present in meat animal glands, organs, blood, and neural tissues (Ansell and Hawthorne, 1964; Wittcoff, 1951). Little attention has been devoted to those in skeletal tissues. Phospholipids of rabbit (Gray and MacFarlane, 1961), sheep (Dawson, 1960), and pork (Hornstein *et al.*, 1961; Kuchmak and Dugan, 1963, 1965) muscle tissues have been isolated and partially identified. Hornstein *et al.* (1961) reported the presence of cephalin, lecithin, and sphingomyelin in beef tissue lipids.

Our interest in polar lipids of skeletal beef tissue stems from the problem of deterioration of freeze-dried meat upon storage and their role in the flavor of processed beef. Lea (1957) has suggested that phospholipids may be involved in detrimental reactions in freeze-dried meat. In cooked meat, phospholipids oxidized more rapidly than the glyceride fraction and produced off-odors very different from those associated with neutral lipid oxidation (Younathan and Watts, 1960). Phospholipid oxidation in model systems has been studied (Koops, 1957), but the degradation products have not yet been identified. The contribution of polar lipids to meat flavor is still obscure, and the fatty acid composition of the individual polar lipids in beef tissue is unknown.

This investigation is part of a program initiated to provide precise information on the identity of beef muscle polar lipids; the fatty acid composition of the individual polar lipids; the role of polar lipids in the flavor of processed beef; and the flavor components present in beef lipids.

This paper describes a method for the extraction of polar lipids from beef muscles and reports the identity of some of the isolated compounds from lipid I.

EXPERIMENTAL

Extraction of Lipids from Muscle (Figure 1). *Semimembranosus* muscle of U. S. Good grade beef round was dissected and trimmed of fat and connective tissues. The muscle was ground twice at ca. 5° C. and homogenized in a Waring Blendor for 4 minutes with an equal amount deionized water. About 1500-ml. aliquots of the slur were transferred into 5-liter round-bottomed flasks, she frozen by immersion and rotation in a dry ice–alcoh bath, and lyophilized. To minimize enzymatic degrad tion and oxidation of lipids, lyophilization was carried o at 5 microns of pressure while the meat slurry was in frozen state. After complete lyophilization, the vacuu was broken with N_2 gas. Lyophilized meat was extracte with petroleum ether (b.p. 30°–60° C.) containing 0.005 hydroquinone in a Soxhlet apparatus for 24 hours. Th solvent was evaporated in a rotary evaporator under vac uum, and the lipid residue was designated lipid I. Th meat residue was extracted twice with deionized water cor taining 0.1% $CHCl_3$ to inhibit tissue phospholipase (Olley and Lovern, 1960) and bacterial growth, and the sub sequent residue was lyophilized, extracted with $CHCl_3$ MeOH, 50 to 50 (v./v.), and $CHCl_3$-MeOH-H_2O, 14:82: (v./v./v.), containing 0.005% hydroquinone. The solvent were removed in a rotary evaporator under vacuum, anc the residues were designated lipids II and III, respectively The extracted lipids were blanketed with N_2 and stored a $-20°$ C.

Column Chromatography of Lipid I. Neutral lipids

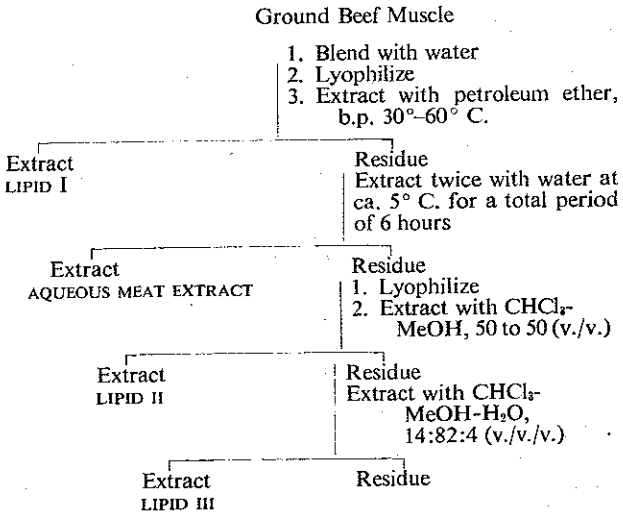

Figure 1. Lipid extraction procedure from beef skeletal tissues

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were separated from the polar lipids by chromatography on activated silica gel, Bio-Sil BH, 100- to 200-mesh (Calbiochem, Los Angeles, Calif.).

A glass column 2.5-cm. i.d. × 50-cm. length was packed with 100 grams of silica gel according to the technique of Hirsch and Ahrens (1958). The eluate flow rate of the resulting column was suitable without the use of a filter aid. The solvents used for elution were reagent grade. The column was washed with 2 liters of chloroform. To obtain good separation, the column load was 20 mg. of lipid per gram of silica gel. Two grams of lipid I were dissolved in $CHCl_3$ and applied to the top of the column. The column was eluted with 2 liters of $CHCl_3$ followed by 2 liters of MeOH. A flow rate of 5 ml. per minute was obtained by applying nitrogen pressure of about 35 mm. of Hg to the top of the column reservoir. Chloroform and methanol fractions were evaporated to dryness in a rotary evaporator under vacuum. Total recovery after fractionation was 99.3 to 100.8%.

Fractionation of Polar Lipids. Total polar lipids rather than acetone-insoluble lipids were investigated to ensure that polar lipids with appreciable acetone solubility were not overlooked.

An anion exchange diethyl aminoethyl (DEAE) cellulose column was prepared from Cellex D, DEAE-cellulose (Calbiochem, Los Angeles, Calif.) with an exchange capacity of 0.9 ± 0.005 meq. per gram, according to the technique of Rouser *et al.* (1961, 1964). Thirty grams of DEAE-cellulose gave a column height of 47 cm. in a glass column, 2.5-cm. i.d. × 50-cm. length. A flow rate of 2 ml. per minute was obtained by applying nitrogen pressure of about 50 mm. of Hg to the reservoir. A sample of 22 mg. of polar lipid per gram of dry DEAE-cellulose was used for loading the column. The sample was added to the column in ca. 20 ml. of $CHCl_3$. Elution was carried out according to the scheme shown in Table I. Successive solvents (Rouser *et al.*, 1961) were applied just as the last of the preceding solvent entered the column.

Column effluent was collected in 5-ml. aliquots which were monitored on silica gel G plates. By combining appropriate aliquots, 17 fractions were obtained. Eluates containing acetic acid were neutralized with $NaHCO_3$ and saturated with NaCl; the chloroform layer was freed from water with anhydrous sodium sulfate. All fractions were concentrated to 8 ml. in a rotary evaporator under vacuum, tested with thin-layer chromatography (TLC), transferred to Teflon centrifuge tubes, evaporated to dryness, and weighed. The contents were blanketed with N_2 and kept at −20° C. TLC indicated the isolated fractions were stable under these conditions up to six months.

Table I. DEAE-Cellulose Chromatographic Elution Scheme[a]

Solvent	Volume, Ml.	Components Eluted
$CHCl_3$-MeOH, 9 to 1	2000	Lyso PC, PC, Sp
$CHCl_3$-MeOH, 7 to 3	3150	PE
MeOH	1500	Wash
$CHCl_3$-HAc, 3 to 1	2000	Triphosphoinositides?
$CHCl_3$-HAc, 3 to 1 + 0.05*M* NH_4Ac	2850	PS, PI
HAc	1500	Uncharacterized compounds
$CHCl_3$-MeOH, 4 to 1 + 0.01*M* NH_4Ac + 20 ml. of concd. aqueous NH_3 per liter	1725	Su?

[a] 0.66 gram of beef polar lipid, column 2.5-cm. i.d. × 47-cm. height.

Thin-Layer Chromatography. Silica gel G plates, 20 × 20 cm., precoated (ca. 250-micron thickness) and prescored (Analtech, Wilmington, Del.) were used in this work. The plates were washed overnight in developing solvent, $CHCl_3$-MeOH-H_2O mixture, 65:25:4 (v./v./v.), activated by heating for 1 hour at 110° C., and stored in a desiccator over $CaSO_4$ until used. Samples and reference compounds, dissolved in methanol, were applied to the plates by means of 5-μl. micropipets. The amount of reference polar lipids and samples applied ranged from 5 to 25 μg. Single development was carried out in $CHCl_3$-MeOH-H_2O, 65:25:4 (v./v./v.). The developing time for a 10-cm. chromatogram was about 25 minutes. For general staining, both exposure to iodine vapors and spraying with Rhodamine 6G and viewing under ultraviolet light proved to be satisfactory. Ninhydrin and molybdenum spray reagents were prepared according to Dittmer and Lester (1964) for the identification of free α-amino nitrogen and phosphate ester groups, respectively.

To verify the identity of the fractions, they were re-examined by TLC using the spiking technique developed by Bobbitt (1967).

Identification was achieved by spray reagents, according to Dittmer and Lester (1964), R_f and infrared of intact polar lipid fractions, and by comparison with reference samples of cerebrosides (Cb), ceramides (Cd), lysophosphatidylcholine (lyso PC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (Sp), and sulfatide (Su).

Infrared Spectrophotometry. Infrared spectra of fractions and reference compounds were recorded in the range of 2.5 to 15 microns with a Perkin-Elmer IR-21 double beam recording spectrophotometer. Potassium bromide disks were prepared by adding 1 mg. of solid sample to 100 mg. of spectrograde KBr. Recorded spectra from KBr disks were used for identification of polar lipids as they were not obscured by solvent absorption.

Acid Hydrolysis of Fraction XIII (of Table IV). Hydrolysis conditions of fraction XIII were those of Taylor and McKibbin (1953). Ten milliliters of chloroform-methanol, 3 to 2 (v./v.), was satisfactory for dissolving and transferring 20 mg. of fraction XIII to a hydrolysis tube. Equivalent sample weights of both pure inositol and PI were run to provide reference standards. Five milliliters of 4*N* HCl were added to each sample; the tubes were evacuated and flushed with nitrogen gas three times before final evacuation for hydrolysis. The tubes were immersed in a glycerol bath at 105° C. for 40 hours. After cooling, the liberated free fatty acids were removed by extracting three times with 15 ml. of petroleum ether. The petroleum ether extracts were combined, washed with water, evaporated to dryness in a rotary evaporator under vacuum, and kept at −20° C. under a blanket of nitrogen. The petroleum ether extract of pure inositol left no residue after evaporation. The aqueous layer of each sample was com-

bined with the aqueous washings of the free fatty acids, concentrated to about 2 ml. in a rotary evaporator under vacuum. Each sample was put in a desiccator containing P_2O_5 and KOH pellets, subjected to vacuum for 4 hours, and left overnight at 5° C. To remove the last traces of HCl, the samples were redissolved in ca. 5 ml. of water, replaced in the desiccator, and subjected to vacuum for 18 hours. The dry powder residues were free of HCl and tested for inositol according to the method of Feigl (1960).

RESULTS AND DISCUSSION

There is no general procedure for lipid extraction from biological sources. Each method depends upon the type of biological material and the nature of the problem. Lyophilization is advantageous as it renders the tissues more permeable to solvents employed in subsequent extraction. Since we are interested in lipid-soluble flavors, polar lipids, and water-soluble flavor precursors of beef, the scheme shown in Figure 1 was planned to fulfill the required goals. This procedure resulted in one extract (lipid I) rich in neutral lipids while the other two extracts (lipids II and III) are rich in polar lipids. Table II shows the lipid fractions extracted from *semimembranosus* muscle.

Lyophilization of homogenized slurried beef muscles resulted in a product of fine structure and more permeable to solvents. Petroleum ether extracted the "free lipids"—i.e., lipids not associated with beef proteins—while chloroform-methanol and chloroform-methanol-water extracted lipids combined with tissue proteins and/or inorganic cations. Aqueous extraction of beef flavor precursors before chloroform-methanol extractions eliminated the possibility of contaminating the polar lipids with carbohydrates, peptides, and amino acids which are solubilized in these solvents in the presence of polar lipids. Baer *et al.* (1952, 1956) reported that 5% solutions of PC in anhydrous and ethanol-free $CHCl_3$ solubilized glucose or sucrose in amounts that were equal to 3.5 or 2.3%, respectively, while sodium chloride was just perceptibly soluble. Furthermore, the aqueous extraction of the flavor precursors before chloroform-methanol extraction eliminated the possibility of denaturation of flavor precursor components and kept them intact.

Extraction with $CHCl_3$-MeOH-H_2O increased the lipid yield by 3%; the additional lipids were strongly bound to beef tissue components. The high efficiency of extraction with this solvent may be attributed to the decrease in the dissociation constant of the acidic polar lipids due to solubilization of the naturally occurring Ca, Mg, K, and Na cations in meat (Watt *et al.*, 1963). Identification of the individual components of lipids II and III will shed light on this assumption.

The extraction procedure of beef lipids presented in this paper can be incorporated as an integral part of meat flavor component studies, as it is characterized by simplicity and uniformity of techniques; nondestruction of extractable materials; exclusion of nonlipid contaminants that would interfere with chromatography of lipids (Some contaminants appear to aid the spontaneous decomposition and oxidation of lipids. The presence of nonlipid materials in the extract gives a total weight which contributes to misleading recovery figures.); and avoidance of protein denaturation which would make subsequent separation of lipids almost impossible.

Polar lipids resulting from fractionation of lipid I on silica gel amounted to 1.7%.

Fractionation of polar lipids on DEAE-cellulose gave 17 fractions. To examine these fractions, the purest polar lipids available were used as standards. Table III shows

Table II. *Semimembranosus* Extractable Lipids

Lipid	Solvent	Fresh Meat, Wt. %	Total Lipids, Wt. %	Aroma
I	Petroleum ether, b.p. 30°–60° C.	3.5	83.54	Tallowy
II	$CHCl_3$-MeOH, 50 to 50 (v./v.)	0.57	13.60	Pound cake
III	$CHCl_3$-MeOH-H_2O, 14:82:4 (v./v./v.)	0.12	2.86	Pound cake

Table III. TLC Properties of Reference Polar Lipids

Compound	R_f[a] I_2	R_f[a] Rh. 6G[b]	R_f[a] Rh. 6G[c]	Color, Rh. 6G: Without ultraviolet light	Color, Rh. 6G: Under ultraviolet light	Spot Test: Ninhydrin	Spot Test: Mo[d]
Monogalactosyl diglyceride	0.85	0.85	0.81	Faint pink	Pink	−	−
Cb	0.67, 0.62	0.70, 0.65	0.72, 0.68	Faint pink	Pink	−	−
Cd	0.92	0.90	0.87	Faint pink	Pink	−	−
Su	0.25	0.30	0.33	Pink	Purplish pink	−	−
Sp	0.14	0.13	0.10	Pink	Fluorescent yellow	−	+
PI	0.06	0.06	0.03	White	Pinkish blue	−	+
PC	0.24, 0.14, 0.08	0.26, 0.15, 0.06	0.26, 0.10, 0.03	Pink	Fluorescent yellow	−	+
Lyso PC	0.08	0.06	0.03	Pink	Fluorescent yellow	−	+
PE	0.39	0.48	0.52	Pink	Yellowish pink	+	+
PS	0.09	0.10	0.07	White	Blue	+	+

[a] R_f values are obtained by TLC on silica gel G plates.
[b] Spraying with Rhodamine 6G.
[c] Spraying with Rhodamine 6G followed by ninhydrin spray.
[d] Molybdenum spray for phosphate test.

the TLC properties of the reference polar lipids.

The reference PC contained two components as impurities; lyso PC was the major one. Also, the Cb sample contained some impurities. As polar lipids vary in their mobilities, R_f values alone are not adequate for identification of unknown; so it is necessary to compare the mobility of the fraction component with those of authentic samples which are run on the same chromatogram and to rely on specific tests. Rhodamine 6G was a valuable asset in identifying the polar lipids on the developed chromatograms.

Under ultraviolet light PS and PI ("acidic" polar lipids) appeared blue, while PC, lyso PC, and Sp ("neutral" polar lipids) gave a fluorescent yellow color. Under the same conditions monogalactosyl diglyceride, Cb, and Cd (unionizable polar lipids) were pink; PE (zwitterion) appeared yellowish pink, while sulfatides (containing galactose sulfate) were violet pink.

Figure 2 represents a tracing of a thin-layer chromatogram of fractions I to VII, XI, and XIII obtained from a DEAE-cellulose column. PS gave a comet streaking chromatogram on TLC because it is eluted as an ammonium salt. Application of the spiking technique lead to the qualitative identification of the polar lipids. Figure 3 demonstrates the application of the spiking technique to fractions I and V.

In Table IV, some analytical data, R_f values, and weights of the 17 fractions are given.

Table IV shows that fractions II, III, and IV are heterogeneous in composition, indicating that PC, lyso PC, and Sp are inseparable on DEAE-cellulose, while fractions I, V, VI, VII, XI, and XIII show only one spot by TLC. Fraction XVI from one of three DEAE-cellulose chromatography runs was resolved into three components by TLC.

Although the infrared spectra of the major polar lipids have been reported in the literature, little has been done to correlate these spectral findings. To interpret the absorption bands which are not assignable to localized configurations of atoms, direct comparisons are made with the spectra of the known major polar lipid moieties (Daasch and Smith, 1951; Neely, 1957; Turvey *et al.*, 1967; Wickberg, 1958). The spectrum of functional groups serves not only for identification, but also to indicate whether or not the compound in question is pure or contains minor components as impurities.

The spectra of both PC and lyso PC of fractions II, III, and IV show a strong band at 5.8 microns characteristic of ester carbonyl, which is absent in sphingomyelin. While lyso PC shows a weak absorption band at 6.1 microns, there is less indication of absorption at this wavelength in PC. On the contrary, PE (fractions V, VI, and VII) and PS (fraction XI) exhibit absorption at 6.1 microns, but this absorption is more intense than that observed in lyso PC and less than Sp. PC, lyso PC, and Sp exhibit strong absorption in the region from 10.20 to 10.40 microns, while PE and PS spectra are void of such absorption. PE and PS which exhibit similar spectra can be identified in the presence of one another by an absorption at 4.7 and 6.25 to 6.40 microns, respectively. Sp in fractions II, III, and IV showed the expected 6.1 microns (amide I band), 6.45 microns (amide II band), and 10.3 microns (C=C trans in sphingosine and P-O-C). Fraction XIII is characterized by a strong absorption band at 3.00 microns due to the free

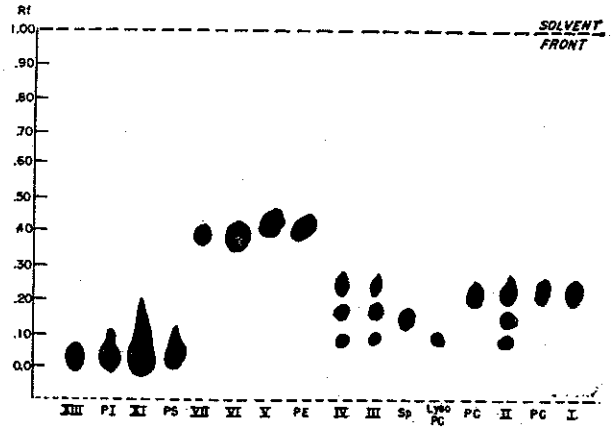

Figure 2. Tracing of chromatogram of beef polar lipid fractions obtained from DEAE-cellulose column

I = PC; II, III, and IV = PC, lyso PC, and Sp; V, VI, and VII = PE; XI = PS; XIII = PI

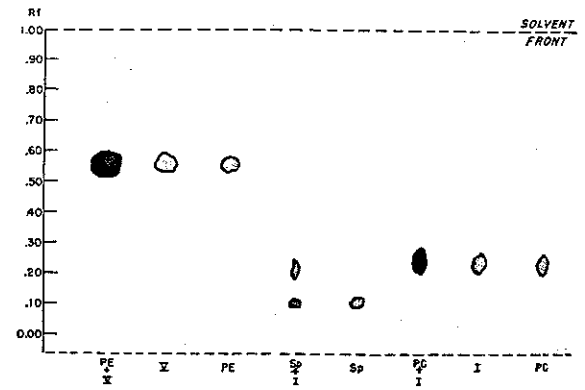

Figure 3. Tracing of chromatogram of fractions I and V upon application of the spiking technique

PC + I (equal to 2.5 μl. of PC + 2.5 μl. of I) gave an oval spot verifying the identity and homogeneity of fraction I; Sp + I (equal to 2.5 μl. of Sp + 2.5 μl. of I), two spots indicating the absence of Sp from fraction I; PE + V (equal to 2.5 μl. of PE + 2.5 μl. of V), one oval spot indicating purity of fraction V

OH in inositol and absorption bands at 11.17, 11.25, and 13.3 microns typical of inositol. Also the products of hydrolysis of this fraction gave a positive test for inositol. TLC showed only one spot with an R_f value of 0.06 similar to that of pure PI; the spiking technique confirmed its identity.

Fraction XVI showed absorption bands at 6.1 and 6.5 microns that are characteristic of a monosubstituted amide; a strong band at 10.3 microns due to C=C trans; and absorption bands at 6.9 to 7.4 and 8.1 microns (S-O stretching), at 10.8 microns (C-O-S present in carbohydrate sulfate), and 11.5 microns (C-H other than anomeric in galactose). On the basis of evidence accumulated from infrared spectra, sulfatide is proposed as a constituent of fraction XVI. Because of the small weight of this fraction, further chemical identification was not feasible.

Figure 2 indicates the presence of significant amounts of lyso PC in fractions II, III, and IV. It is not an artifact as the lipids were not subjected to any treatment that would lead to its formation. Lyso PC in beef polar lipids may be a normal constituent of an enzymatic or nonenzymatic deg-

Table IV. Polar Lipid Composition of *Semimembranosus*

Solvent	Fraction No.	Vol., Ml.	Wt., G.	Wt. %	Ninhydrin[a]	Mo[b]	Proposed Constituents
$CHCl_3$-MeOH, 9 to 1	I	12	0.00903	1.3	−	+	PC
	II	90	0.26242	37.0	−	+	PC, lyso PC, Sp
	III	18	0.01123	1.6	−	+	PC, lyso PC, Sp
	IV	108	0.01514	2.1	−	+	PC, lyso PC, Sp
	V	852	0.08762	12.4	+	+	PE
$CHCl_3$-MeOH, 7 to 3	VI	390	0.09801	13.8	+	+	PE
	VII	699	0.01998	2.8	+	+	PE
MeOH	VIII	140	0.01601	2.3	−	−	Uncharacterized
$CHCl_3$-HAc, 3 to 1	IX	20	0.00879	1.2	−	+	(Triphosphoinositide)?
	X	85	0.01132	1.6	−	+	(Triphosphoinositide)?
$CHCl_3$-HAc, 3 to 1 + 0.05M_3 NH_4Ac	XI	45	0.04510	6.4	+	+	PS
	XII	215	0.01532	2.2	−	−	Uncharacterized
	XIII	345	0.02139	3.0	−	+	PI
HAc	XIV	60	0.02535	3.6	−	−	Uncharacterized
$CHCl_3$-MeOH, 4 to 1 +	XV	170	0.02311	3.3	−	−	Uncharacterized
0.01M NH_4Ac + 20 ml. of	XVI	90	0.00718	1.0	−	−	Uncharacterized (Su?)
concd. NH_4OH per liter	XVII	1060	0.02953	4.2	−	−	Uncharacterized

[a] Ninhydrin spray for amino nitrogen test.
[b] Molybdenum spray for phosphate test.

radation product in muscles before extraction. The presence of Sp, PC, and PE in beef muscle is in agreement with the Hornstein *et al.* findings (1961).

Quantitative column chromatographic analysis of petroleum ether–extractable polar lipids gave the following results (weight per cent): PC, lyso Pc, and Sp, 42.0; PE, 26.4; PS, 6.4; PI, 3.0; unidentifiable lipids, 22.2; and traces of sulfatide.

For the first time, PI, which has previously been reported in fish muscle (Lovern, 1956), was isolated from skeletal beef tissue. Work is in progress to identify the individual components of lipids II and III.

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